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The frequency of occurrence of human fungal infections is increasing in response to a combination of factors including advances in invasive surgical techniques allowing for pathogen access, immuno-suppression employed in transplantation or resulting from chemotherapy, disease states such as AIDS, and the increase in resistance to the currently available antifungal drugs. The serious problem of resistance impacts both systemic fungal infections as well as topical infections such a yeast vaginitis which is widespread and results in significant loss of work time and efficiency. The research reported here describes advances made in the identification of potential new sites in fungal biosynthesis for the development of novel antifungal compounds. The *ERG6* gene of the human pathogen, *Candida albicans*, has been isolated, sequenced and disrupted in this organism. The resulting *erg6* strain is viable but is compromised in several important functions including the ability to limit or exclude the entry of exogenous substances. This trait is now being more thoroughly characterized to assess increased sensitivity to antifungal compounds. A second *Candida* gene which encodes the C-4 sterol methyl oxidase (*ERG25*), has also been isolated and sequenced. Disruption experiments are now underway to determine if this gene is essential for viability in this organism.

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Martin Band 9/10/97

N Douglas Lees 9/10/97

PI - Signature Date

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INTRODUCTION

Subject: The research supported by this award is designed to address the need for the discovery and development of new classes of antifungal drugs. As eucaryotic organisms, fungal pathogens provide limited targets for inhibitory or lethal compounds that do not also have deleterious effects on the host. One target that has been exploited successfully in the development of antifungal compounds is the sterol biosynthetic pathway. The drugs that are currently available for intervention in fungal sterol biosynthesis or function are becoming less effective thus making the search for new compounds and sites of action mandatory. Our work proposes to explore previously uncharacterized steps in sterol biosynthesis as possible new sites for new drug discovery. The specific steps in our approach will utilize primary discovery in *Saccharomyces cerevisiae* to address the largely unexplored pathway in *Candida albicans*, a common fungal pathogen.

Purpose: The major purpose of this work is to address the critical rise in fungal infections noted over the last decade (1). A significant amount of the increase has been due to medical advances in other areas that permit entry of opportunistic fungal pathogens into the human body. Included are new surgical procedures and technologies for artificial structural replacements, increased incidence of organ transplants made possible by the development of methods to disarm the immune system, and chemotherapies which are immunosuppressive. In addition, the rise in disease states, especially the situation posed by HIV, has added to the overall problem.

The array of antifungal compounds currently available to deal with this problem is limited and of decreasing efficacy. This has spurred intensive research in the development of new compounds. The primary classes of drugs in use include the polyenes and the azoles. The former have been in use for many years and function by direct interaction with membrane sterol. The result of this interaction is the formation of pores through which leakage of intracellular components occurs leading to cell death (2). Specificity for fungal cells is conferred by the enhanced binding efficiency to ergosterol, the fungal membrane sterol, compared to cholesterol. The binding specificity, however, is not absolute and interaction with host membrane sterol does lead to serious side effects. For example, amphotericin B, a systemically employed polyene, is toxic to renal tissue. Coupled with the emergence of resistance (3, 4), these side effects limit the use of this group of drugs.

The azole antifungals are a newer class of antifungals that are available in many species and in formulations for topical and systemic applications. These drugs are fungistatic thus making their effectiveness in situations where the host is immuno-compromised problematic over the long term. Azoles function by inhibiting the cytochrome P450-mediated removal of the C-14 methyl group from the ergosterol precursor, lanosterol. The azoles are significantly more active against the fungal enzyme as compared to the human version of the C-14 demethylase.

Widespread use of azoles for a number of fungal infections has led to significant and increasing incidence of resistance (5, 6). Within the last two years alone there have been many reports in the literature documenting azole resistance in *C. albicans* (7-11) as well as in other species of *Candida*(12, 13). Furthermore, resistance has also appeared in several other pathogenic fungi including species of *Crytococcus* (14, 15), *Histoplasma* (16), and *Aspergillus* (17). Recent work in *C. albicans* has defined the mechanisms by which resistance occurs. Mutations in and increases in expression of the gene (*ERG11*) for the C-14 demethylase enzyme, the azole target, have been implicated in some resistant isolates (8, 11). Also, over expression of the small molecule efflux systems such as the ABC (ATP-binding cassette) transporter family and major facilitator family of pumps (7, 9, 10) have been identified as resistance mechanisms. Thus, it appears that the efficacy of the azoles is diminishing and that the arsenal of available antifungals will be further reduced in many infections.

Of particular interest to the US Army Defense Women's Health Research Program, are fungal infections unique to women. The most common such afflictions are vaginal infections caused primarily by *C. albicans*. Vaginal yeast infections will occur in three quarters of the female population at least once during their lifetime (18). Although not considered life-threatening, this infection results in sufficient discomfort that time from work and lost efficiency frequently result. This condition is exacerbated under conditions where medical attention is not readily available and extended stays in the field are required. The most often prescribed treatments for yeast vaginitis are polyene and azole drugs. Also of interest to the Army, although not specific to women, are deep tissue infections caused by accidents or wounds and burns, both of which are commonly colonized by opportunistic fungal pathogens.

Scope: The basic approach of this research is to identify steps in sterol biosynthesis that might be new targets for the development of novel antifungal compounds. This work closely follows our work in *S. cerevisiae*, which though not a pathogen, is an excellent model system for eucaryotic cell studies, in general, and sterol biosynthesis studies, in particular. To that end we have isolated and characterized the genes of many of the steps in the yeast sterol pathway. One of those steps, the transmethylation at position C-24 (*ERG6*), is of particular interest to us and further exploration of this gene in *C. albicans* is one of the aims of this project.

Mutations in the *ERG6* gene of *S. cerevisiae* were originally isolated based on resistance to nystatin, a polyene antibiotic (19). Although these mutants were viable there was speculation based on early growth experiments (20) that the *ERG6* step might be essential for the cell. If this were the case, it would make an excellent target for drug development since this step is not found in cholesterol biosynthesis thus eliminating one potential side effect problem. This possibility could not be fully explored until the *ERG6* gene was cloned and disrupted since the mutations originally isolated could be leaky and allow some ergosterol synthesis. The question of the essentiality of the *ERG6* gene was resolved by Gaber et al. (21) who showed

that *ERG6* disrupted strains were viable although loss of the *ERG6* gene product resulted in several altered phenotypes. Many of these phenotypes were consistent with altered physiological (22, 23) and membrane structural features (24, 25) that we have described previously in *erg6* mutants. Summarizing these findings, *erg6* mutants are compromised in several physiological areas (growth limitations on a variety of energy sources, mating difficulties, low transformation frequency, permeability alterations) due to the presence of membrane sterol that does not have the C-24 methyl group (21-25).

The most important of the alterations noted in *erg6* cells was the increased permeability of the cell to a variety of substances. This trait was demonstrated in our lab using a variety of molecules including dyes (22), cations (22) and spin labels used in electron paramagnetic resonance studies (24). Increased permeability was also employed in the cloning strategy (21) for the *ERG6* gene and, subsequently, several other pathway genes. More recently, cloning of the *LIS1* gene, mutations of which lead to increased uptake of lithium and sodium, was accomplished and sequencing revealed that this gene was identical to *ERG6* (26). Our hypothesis is that disruption the *ERG6* gene of *C. albicans* or inhibition of the *ERG6* gene product in this organism would generate a phenotype similar to that seen in *S. cerevisiae ERG6* disruptants. Such a phenotype would make the cell more sensitive to known antifungals and more permeable to inhibitory substances that would not normally be able to gain entry to the cell. We would also be interested in determining the effect of the *erg6* mutation on the ability of *C. albicans* to form hyphae, a property necessary for pathogenesis. We have shown previously that a mutation in the *ERG11* step seriously compromises the ability of this organism to form hyphae (27). With these possibilities in mind, we are seeking to disrupt both copies of the *C. albicans ERG6* gene, test it for increased sensitivity to a variety of substances, and utilize the isolated *ERG6* gene in a screen for the isolation of substances that block the function of the *ERG6* gene product.

Most recently (28) we isolated and characterized the *S. cerevisiae* gene (*ERG25*) encoding the C-4 sterol methyl oxidase, the enzyme responsible for initiating the removal of the two methyl groups from the C-4 position. This work has leveraged our ability to isolate the *ERG25* gene from *C. albicans*. Since we have determined that the *ERG25* gene is essential to yeast for survival, verification of this phenotype in *C. albicans* might indicate that the *ERG25* gene product would be a good candidate target for the development of new antifungals. We now have the complete sequence of the *C. albicans ERG25* and are in the process of disrupting both copies to determine if it is essential in this organism as it is in *S. cerevisiae*.

We are also interested in the phenomenon, noted in *S. cerevisiae*, where essential genes in the sterol biosynthetic pathway can be suppressed by mutations in other genes and viability is thus restored. Defining such mechanisms will help determine and analyze such events should they occur in *C. albicans*.

Background: Sterols are required components of all eucaryotic membranes and numerous studies of the physiological and biophysical effects of alteration of membrane sterol content have indicated their importance for normal permeability functions(23-25). In addition, sterols play a critical role in maintaining the appropriate membrane viscosity such that membrane bound enzymes can perform normally (29). Our best information on ergosterol biosynthesis comes from studies using the common yeast, *S. cerevisiae.*

Sterols are synthesized as a separate branch of the isoprenoid pathway which also produces essential end products such as quinones, heme, and dolichols (30). In addition, intermediates such as farnesyl pyrophosphate and geranyl pyrophosphate are employed in the isoprenylation of proteins that require membrane anchoring for appropriate functioning (30).

The sterol branch of the isoprenoid pathway is shown in Figure 1. The first compound, squalene, is formed by the enzyme squalene synthase by combining two molecules of farnesyl pyrophosphate. The next two reactions yield lanosterol, the first sterol in the pathway. Mutations in any of the steps prior to lanosterol formation result in sterol auxotrophy and cells carrying such mutations must be provided exogenous sterol. Mutations in any of the three steps (*ERG11, ERG 24,* and *ERG25*) following lanosterol synthesis result in aerobic non-viability since cells producing sterol can no longer take up exogenous sterol and these mutants produce sterol intermediates that cannot be utilized in place of ergosterol(31, 32, 28). Mutations in the subsequent steps of the pathway have been shown to produce sterol intermediates capable of supporting growth. These mutants, however, do show altered phenotypes with those furthest from the ergosterol end product showing the most compromised characteristics.

Our knowledge of the sterol biosynthetic pathway in the pathogen, *Candida albicans,* is considerably less complete. The same basic steps are present although the order of some of the terminal reactions is altered compared to *S. cerevisiae.* The determination of essentiality for various steps in *S. cerevisiae* has been accomplished using gene disruption and allele replacement techniques. Since only a handful of genes in *C. albicans* have been cloned, much remains to learned about the essential reactions of the pathway in this organism.

In addition to characterization of the ergosterol biosynthetic steps in *C. albicans,* it is important that the phenomenon of genetic suppression be explored. Suppression occurs when a second mutation masks the effects of the primary mutation. In the case where the first mutation is lethal, the suppressor would result in viability. This is critical consideration if we are able to identify an essential reaction and design an inhibitor only to find that a suppressor mutation counteracts the inhibitor.

Suppression has been identified in all *ERG11, ERG24,* and *ERG25* steps of ergosterol biosynthesis in *S. cerevisiae.* Mutations in the *ERG11* gene are suppressed

by downstream mutations in the *ERG3* gene. This is possible because blocks at *ERG11* result in C-14 methylsterols that are acted upon by downstream enzymes. However, the enzyme encoded by the *ERG3* gene cannot complete the desaturation of the C-14 sterol intermediate resulting in the formation of a toxic sterol diol (33). Mutations in *ERG3* result in no diol formation and permit viability in *erg11* mutants (34). Mutations in the *ERG24* gene result in the accumulation of ignosterol, a sterol that cannot support growth. However, a mutation, *fen1* (35), can suppress *erg24* and permit the cell to grow on ignosterol. The nature of *fen1* remains undefined. Finally, we have recently reported (36) on a novel mechanism of suppression of a mutant of the *ERG25* gene. This suppression involves two separate mutations. One is a mutation in *ERG11* while the second is a leaky mutation in one of the heme biosynthetic genes resulting in low levels of heme.

Suppression in the sterol pathway has not been explored in *C. albicans*. It is curious to note that *ERG11* mutations in *C. albicans* are viable and suppression is not required to permit growth. This means that *C. albicans* can either tolerate the sterol diol that accumulates (31) or that this organism is able to selectively sequester this molecule. Thus, sterol requirements and suppression may vary between *S. cerevisiae* and *C. albicans*. Both of these parameters will be addressed during the course of our studies.

BODY

Experimental Methods and Results:

CLONING AND SEQUENCING OF THE *CANDIDA ALBICANS ERG6* GENE

Previously, we reported that the *C. albicans ERG6* gene was cloned using a *Candida* gene library transformed into a *S. cerevisiae erg6* strain. We obtained two complementing and overlapping clones with inserts of 7.8 kb and 14 kb. pIU885 contains a 2.4 kb *Xba*I-*Eco*RI *Candida* DNA fragment which is able to complement the *Saccharomyces erg6* mutation (Figure 2). This DNA fragment was sequenced using the Sanger dideoxy chain termination method and the DNA and amino acid sequences are presented in Figure 3. The *Candida ERG6* gene encodes the sterol methyl transferase which contains 377 amino acids and is 66% identical to the *Saccharomyces* enzyme. Figure 4 shows an alignment between the *Candida*, *Saccharomyces*, *Arabidopsis*, and *Triticum* sterol methyl transferases and the percent identity of *Candida* to the latter two are 40% and 49%, respectively. A 9 amino acid region (Figure 4; amino acids 129-137) represents the highly conserved S-adenosyl methionine binding site (37).

DISRUPTION OF THE *ERG6* GENE

Disruption of the *Candida ERG6* gene to derive a sterol methyltransferase deficient strain was made more difficult since *Candida*, unlike *Saccharomyces*, is an obligate diploid and thus both copies of the *ERG6* gene must be disrupted. To

accomplish this, we used the "ura blaster" system developed by Fonzi (38). Essentially the ura blaster contains ~1.2 kb repeat elements of *hisG* (derived from *Salmonella*) flanking the *Candida URA3* gene (see Figure 2). A plasmid containing the ura blaster inserted into the *ERG6* gene is shown in Figure 2. The 2.4 kb *Xba*I-*Eco*I *ERG6* DNA fragment was cloned into the Bluescript vector pKS(+) in which a *Hin*dIII site was filled in with the Klenow fragment of DNA polymerase I (pIU886). pIU886-L was subsequently derived by deleting a 0.7 kb *Hin*dIII within the *ERG6* coding sequence, filling in this site with Klenow followed by the addition of *Bam*HI linkers. Plasmid 5921, containing the ura blaster, was digested with *Sna*BI and *Stu*I, both blunt cutting enzymes, followed by religation. This resulted in a deletion of 6 bp in one of the *hisG* regions and destruction of these two sites. The modified 5921 plasmid was then digested with *Bam*HI and *Bgl*II to release the 3.8 kb ura blaster which was then ligated into pIU886-L that had been digested with *Bam*HI to generate pIU887-A, a plasmid containing the ura blaster inserted into *ERG6* (Figure 2).

Candida strain CAI4 was transformed using the 5.3 kb *Bgl*II-*Sna*BI fragment containing the ura blaster and *ERG6* flanking recombinogenic ends of 0.8 and 0.9 kb. Transformants containing the single disrupted *ERG6* allele resulting in heterozygosity for *ERG6* was confirmed using PCR after selection for loss of the *URA3-hisG* region. Intrachromosomal recombination between the linear *hisG* sequences results in loss of one of these *hisG* repeats and the *URA3* thus permitting reuse of the ura blaster for the subsequent disruption of the *ERG6* gene on the homologous chromosome. Selection for colonies on 5-Fluoro-orotic acid results in growth of only uracil requiring strains (39).

CREATION OF A HOMOZYGOUS *erg6* STRAIN

The creation of a *Candida erg6* mutant strain in which both alleles were disrupted was accomplished in two different ways. The *ERG6* heterozygote was placed onto plates containing high concentrations of nystatin (20μg/ml) and after 3 days nystatin resistant colonies appeared. We surmised that mitotic recombination might result in homozygous *ERG6* and *erg6* segregants and these nystatin resistant colonies might be the *erg6* homozygotes. When colony purified, these resistant colonies indeed turned out to be *erg6* homozygotes (see next section). The second method used to generate *erg6* homozygotes was to transform the *ERG6* heterozygote with the ura blaster. This time two kinds of transformants were obtained -wild type and slow growing colonies. Both types of colonies were tested for resistance to nystatin and only the slower growing colonies appeared to be nystatin resistant.

CONFIRMATION OF *erg6* HOMOZYGOSITY

All putative *erg6* mutants generated by both methods were nystatin resistant. The sterols isolated from wild type and putative *erg6* homozygotes were analyzed by UV spectrophotometry and gas-chromatography/mass spectroscopy. Yeast sterol

samples were scanned by UV in the 200-300nm range for absorption maxima at 262, 271, 282, and 293 indicating a conjugated diene system in the B ring of the sterol molecule. Additionally, *erg6* mutants accumulate sterols with a conjugated diene system in the sterol side-chain resulting in absorption maxima at 230 and 238nm (19). All of our putative *erg6* homozygotes contained *erg6*-like sterol profiles. Additionally, GC/MS of *erg6* mutant sterols confirmed that only cholesterol-like (C-27) sterols accumulate since the side-chain cannot be methylated. Figure 5 represents a GC profile demonstrating that the putative *erg6* mutants accumulate C-27 sterols and are deficient in side-chain transmethylation. Whereas the predominant sterol in the CAI4 wild type is ergosterol (peak B, 76%,), the principal sterols in *erg6* mutants are: zymosterol (peak A, 43%), cholesta-5,7,24-trien-3β-ol (peak D, 6%), cholesta-7,24-dien-3β-ol (peak E, 9%), and cholesta-5,7,22,24-tetraen-3β-ol (peak F, 29%). Lastly, PCR analyses using combinations of four different primers within the *ERG6* region and the *hisG* region confirmed that both *ERG6* wild type alleles were disrupted.

CLONING AND SEQUENCING OF THE CANDIDA ALBICANS *ERG25* GENE

Previously, we reported that the *C. albicans ERG25* gene was cloned using a *Candida* gene library transformed into a *S. cerevisiae erg25* strain. Again, we obtained two complementing and overlapping clones with inserts of 6 kb and 3.8 kb. Plasmid pIU870 (see Figure 6) containing a 2.9 kb *Bam*HI-*Bgl*II DNA fragment inserted into pRS316 complemented the *erg25* mutation. Two subclones of pIU870 containing a 0.8 kb *Hin*d III fragment (pIU875) and a 0.6 kb *Hin*dIII-*Bgl*II fragment (pIU876) were used for DNA sequencing (Figure 6). A third subclone containing a 1.5 kb *Bgl*II-*Eco*RI fragment was found not to contain *ERG25* sequence but instead DNA encoding isocitrate dehydrogenase. Figure 7 shows the DNA and amino acid sequences of *Candida ERG25*. The *Candida* sterol methyl oxidase is a 294 amino acid protein having 65% identity with the *Saccharomyces* enzyme and 36% identity with the human enzyme (see Figure 8). Conserved sequences among all three enzymes are three histidine clusters required for binding of non-heme iron (Figure 7, boxed region). Additionally, all three enzymes contain the ER retrieval motif KKXX or KXKXX required for retrieving protein from the Golgi back to the ER (40).

Discussion and Conclusion:

The project has three aims as shown in the Statement of Work (see Appendix). The first aim seeks to explore the *ERG6* gene of *C. albicans* as a potential target for the development of new antifungals. One year ago we reported the cloning of this gene by complementation of an *erg6* mutant of *S. cerevisiae* with a *Candida* library. Sequencing of the *Candida ERG6* gene is now complete (Figure 3). We have also completed disruption of both alleles in this organism and found the *ERG6* to be non-essential for viability. We are now in the process of defining the characteristics of cells harboring the double *ERG6* disruption and preliminary data indicate that removal of *ERG6* gene function results in cells with altered growth

indicate that removal of *ERG6* gene function results in cells with altered growth characteristics and permeability characteristics similar to those described for *erg6* mutants of *S. cerevisiae*.

The second aim of our project seeks to isolate and disrupt the genes responsible for the C-4 demethylation step in ergosterol biosynthesis. This is the final unexplored step in fungal sterol pathway. This effort is tied to our work in *S. cerevisiae* where the initial experimentation must be done. The first phase in C-4 demethylation is an oxidation of the C-4 methyl group. The gene (*ERG25*) responsible for this reaction (sterol methyl oxidase) has been isolated and characterized in our laboratory (28). The *Candida ERG25* has been isolated by complementation of a *S. cerevisiae erg25* mutant with a *Candida* library. Since our last report the *Candida ERG25* gene has been completely sequenced (Figure 7). We are now in the process of disrupting both alleles of *ERG25* in this organism. This is a more difficult process since it is likely that the *erg25* phenotype will not be viable and *Candida* does not take up exogenous sterol to maintain viability. We are devising some protocols that will allow us to perform the disruptions in a background that will permit viability.

The final aim of our project is to explore the phenomenon of suppression in *Candida*. As is the case in the C-4 demethylation aim, we will first define suppression mechanisms in *S. cerevisiae* since this organisms has many genetic and physiological advantages. We have recently described a unique suppression mechanism for *erg25* mutants of *S. cerevisiae* and will investigate suppression of this step in *Candida* once we have created the double disruption.

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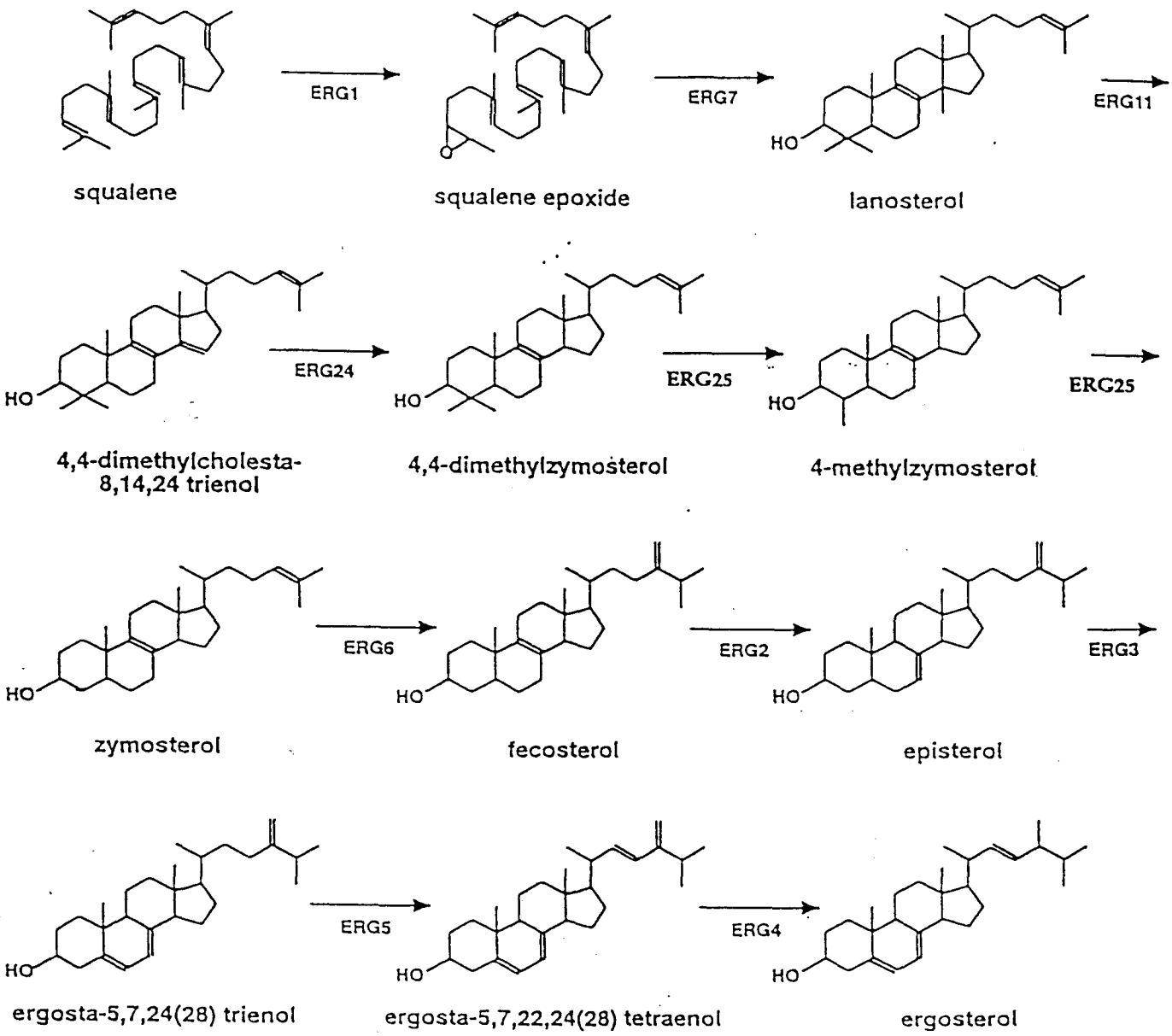

Figure 1. The ergosterol biosynthetic pathway from lanosterol to ergosterol.

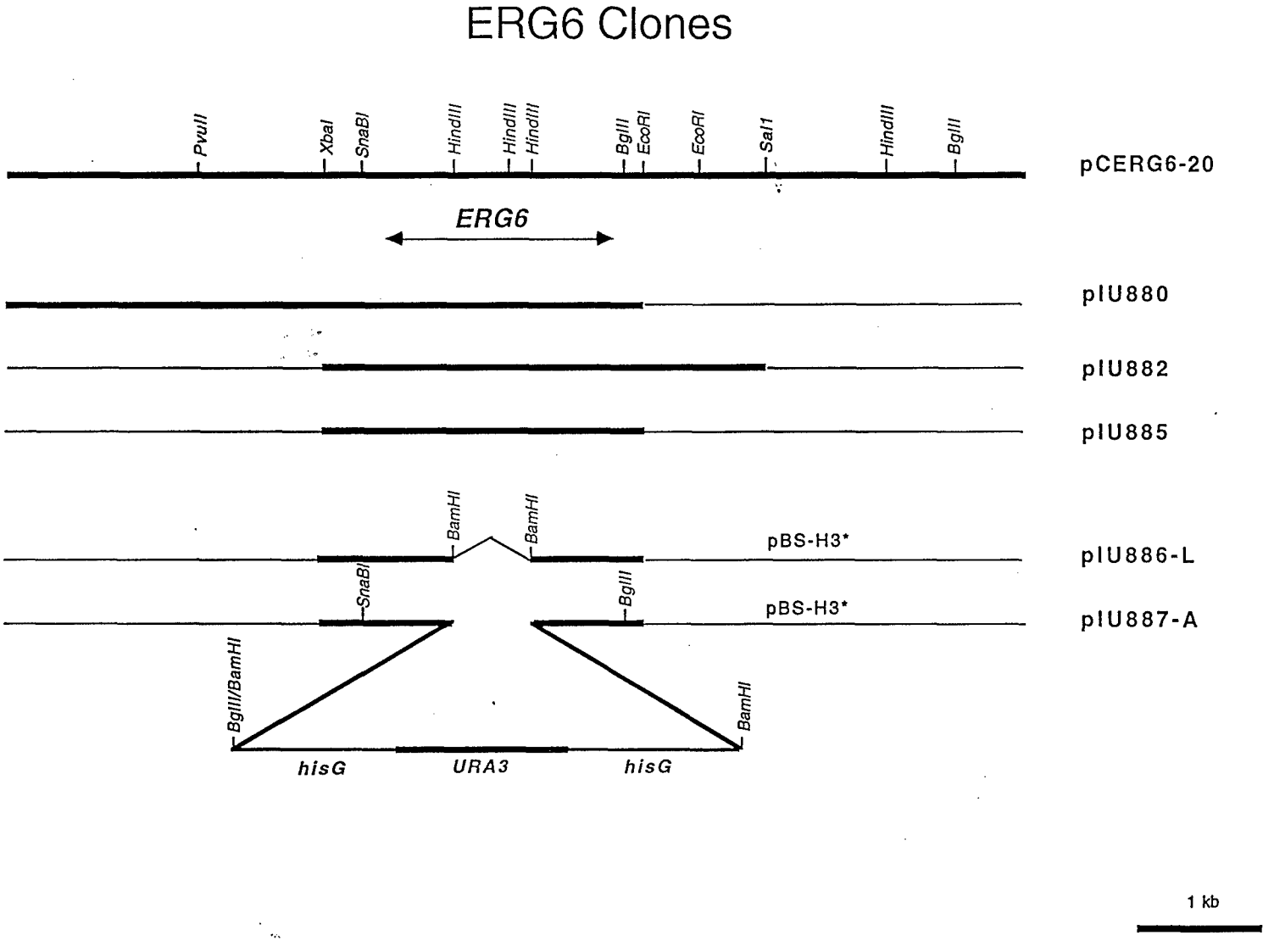

Figure 2. A *Candida albicans ERG6* genomic clone (pCERG6-20) with restriction sites and three complementing subclones, pIU880, pIU882, and pIU885. Modification of pIU885 to pIU886-L and subsequent disruption with the ura blaster shown in pIU887-A are represented.

```
   1  atacaaaatttctttctttccttttttactttacaattatttaaatcaagttaaagaacctttcatttttcttatttta
  80  atttaatttagaaattttcaacctaattaattcataatctaagattcaactcattaaca ATG TCT CCA GTT CAA
                                                                  M   S   P   V   Q     5

 155  TTA GCA GAA AAA AAT TAC GAA AGA GAT GAA CAA TTC ACT AAA GCT TTA CAT GGT GAA TCT
      L   A   E   K   N   Y   E   R   D   E   Q   F   T   K   A   L   H   G   E   S    25

 216  TAT AAA AAA ACT GGG TTA TCA GCT TTA ATA GCT AAA TCT AAA GAT GCT GCT TCT GTT GCT
      Y   K   K   T   G   L   S   A   L   I   A   K   S   K   D   A   A   S   V   A    45

 277  GCT GAG GGT TAT TTC AAA CAT TGG GAT GGT GGT ATT TCT AAA GAT GAT GAA GAG AAA AGA
      A   E   G   Y   F   K   H   W   D   G   G   I   S   K   D   D   E   E   K   R    65

 338  TTG AAT GAT TAT TCC CAA TTG ACT CAT CAT TAT TAT AAT TTA GTC ACT GAC TTT TAT GAA
      L   N   D   Y   S   Q   L   T   H   H   Y   Y   N   L   V   T   D   F   Y   E    85

 399  TAT GGT TGG GGT TCT TCA TTC CAT TTT TCA AGA TAT TAT AAA GGT GAA GCT TTT AGA CAA
      Y   G   W   G   S   S   F   H   F   S   R   Y   Y   K   G   E   A   F   R   Q   105

 460  GCT ACT GCT AGA CAT GAA CAT TTC TTG GCC CAT AAA ATG AAT CTT AAT GAA AAC ATG AAA
      A   T   A   R   H   E   H   F   L   A   H   K   M   N   L   N   E   N   M   K   125

 521  GTT TTA GAT GTT GGT TGT GGT GTA GGT GGT CCT GGT AGA GAA ATC ACA AGA TTT ACT GAT
      V   L   D   V   G   C   G   V   G   G   P   G   R   E   I   T   R   F   T   D   145

 582  TGT-GAA ATT GTT GGA TTA AAT AAT AAT GAT TAT CAA ATT GAA AGA GCT AAT CAT TAT GCT
      C   E   I   V   G   L   N   N   N   D   Y   Q   I   E   R   A   N   H   Y   A   165

 643  AAA AAA TAC CAT TTA GAT CAT AAA TTA TCT TAT GTT AAA GGT GAT TTT ATG CAA ATG GAT
      K   K   Y   H   L   D   H   K   L   S   Y   V   K   G   D   F   M   Q   M   D   185

 704  TTT GAA CCA GAA TCA TTC GAT GCT GTT TAT GCC ATT GAA GCT ACC GTT CAT GCT CCA GTT
      F   E   P   E   S   F   D   A   V   Y   A   I   E   A   T   V   H   A   P   V   205

 765  TTA GAA GGA GTT TAT TCA GAA ATT TAT AAA GTT TTG AAA CCA GGT GGT ATT TTC GGT GTT
      L   E   G   V   Y   S   E   I   Y   K   V   L   K   P   G   G   I   F   G   V   225

 826  TAT GAA TGG GTC ATG ACT GAT AAA TAC GAT GAA ACT AAT GAA GAA CAT CGT AAA ATT GCT
      Y   E   W   V   M   T   D   K   Y   D   E   T   N   E   E   H   R   K   I   A   245

 887  TAT GGT ATT GAA GTC GGT GAT GGT ATT CCA AAA ATG TAT TCT CGT AAA GTT GCT GAA CAA
      Y   G   I   E   V   G   D   G   I   P   K   M   Y   S   R   K   V   A   E   Q   265

 948  GCT TTG AAA AAT GTT GGA TTT GAA ATT GAA TAT CAA AAA GAT TTG GCT GAT GTT GAT GAT
      A   L   K   N   V   G   F   E   I   E   Y   Q   K   D   L   A   D   V   D   D   285

1009  GAA ATT CCT TGG TAT TAT CCA TTA AGT GGT GAT TTG AAA TTT TGT CAA ACT TTT GGT GAT
      E   I   P   W   Y   Y   P   L   S   G   D   L   K   F   C   Q   T   F   G   D   305

1070  TAT TTG ACT GTT TTC AGA ACT TCA AGA ATT GGT AGA TTC ATT ACT ACT GAA TCA GTT GGT
      Y   L   T   V   F   R   T   S   R   I   G   R   F   I   T   T   E   S   V   G   325

1131  TTA ATG GAA AAA ATT GGT TTA GCT CCA AAA GGT TCT AAA CAA GTT ACT CAT GCT TTA GAA
      L   M   E   K   I   G   L   A   P   K   G   S   K   Q   V   T   H   A   L   E   345

1192  GAT GCT GCT GTT AAT TTA GTT GAA GGT GGT AGA CAA AAA TTG TTT ACT CCA ATG ATG TTG
      D   A   A   V   N   L   V   E   G   G   R   Q   K   L   F   T   P   M   M   L   365

1253  TAC GTT GTT AGA AAA CCA TTA GAA AAG AAA GAT TAA tggggcttgacaaacaacaagtaaggtgagtt
      Y   V   V   R   K   P   L   E   K   K   D   *                                   377

1322  tatgttgggggtgttcaattcgtgtatctattcatagaggtatttgatttgcaatttgtttttttgtttattctatttatt
1402  attttaaatactgtttatatgtttttttttaatatgcttagtttctaatagagaaatcaataataatgatcatcatcgtca
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Figure 3. The DNA and amino acid sequences of the *Candida albicans ERG6* gene.

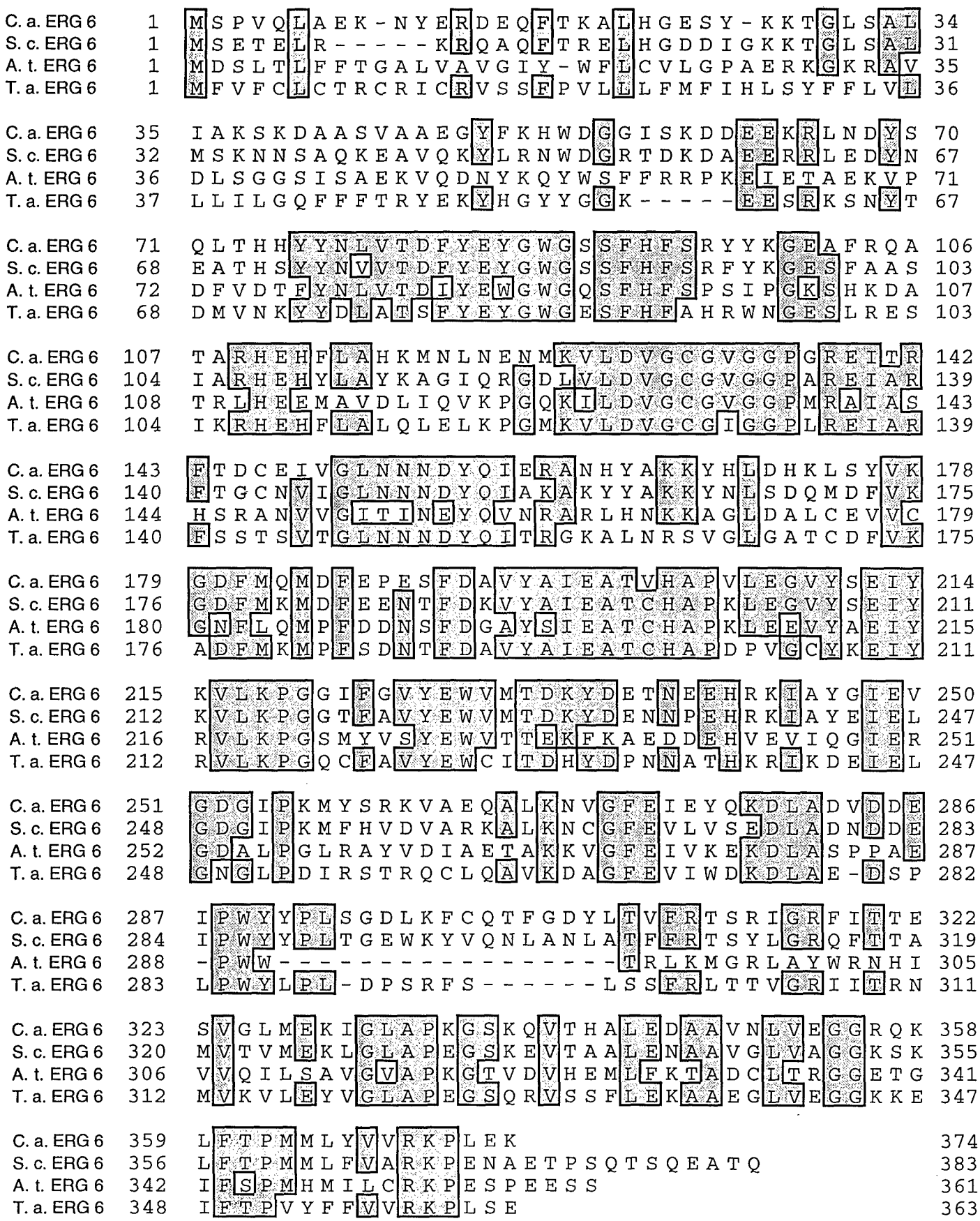

Figure 4. Alignment of the amino acid sequences of the sterol methyl transferases from *Candida albicans* (C. a.), *Saccharomyces cerevisiae* (S. c.), *Arabidopsis thaliana* (a. t.), and *Triticum ativum* (t. a.).

UNPUBLISHED DATA

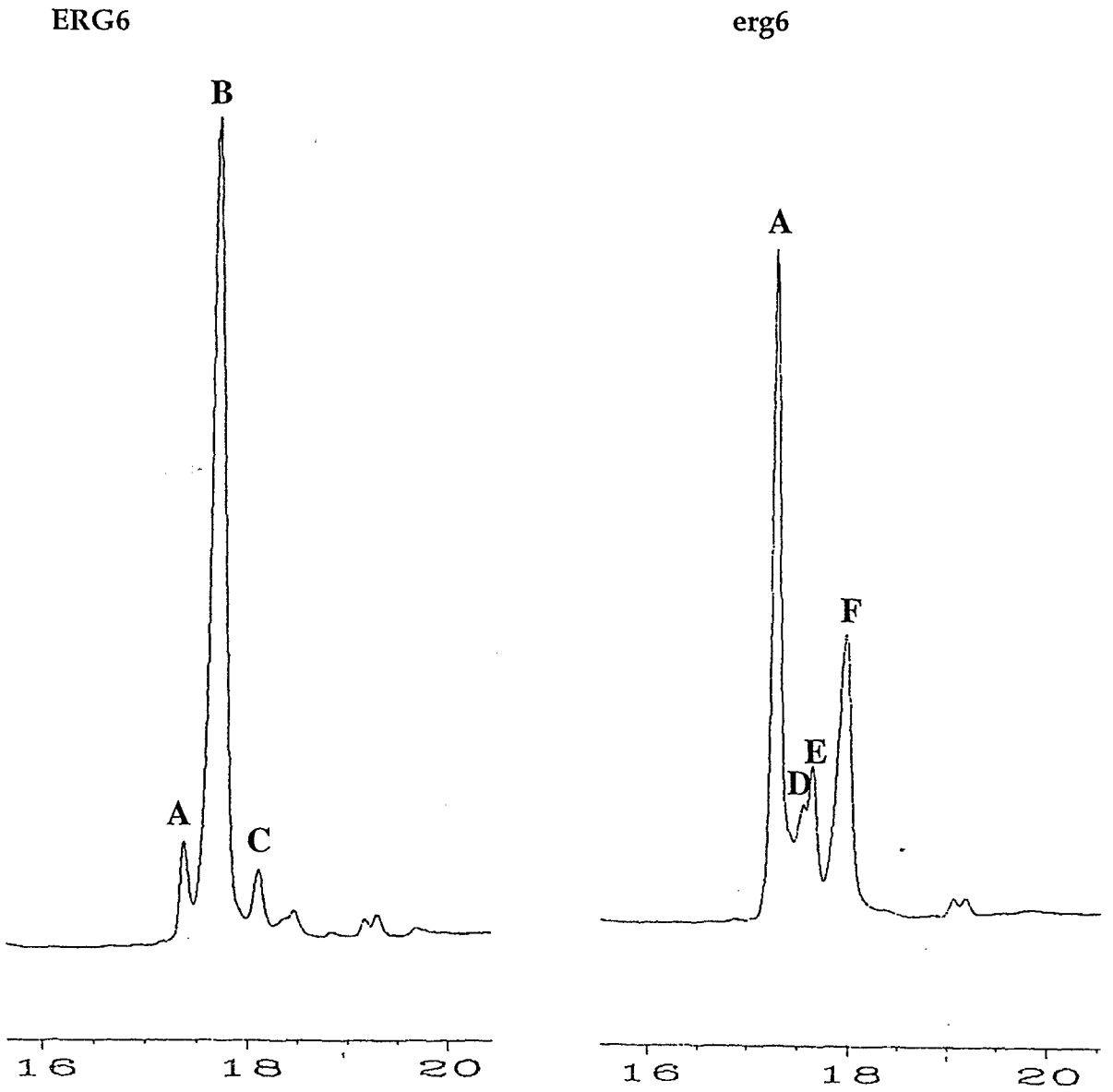

Figure 5. Gas chromatography wild type and an *erg6* strain of *Candida albicans*. Peak A; zymosterol, Peak B; ergosterol, Peak C; fecosterol, Peak D; cholesta-5,7,24-trien-3β-ol, Peak E; cholesta-7,24-dien-3β-ol, Peak F; cholesta-5,7,22,24-tetraen-3β-ol.

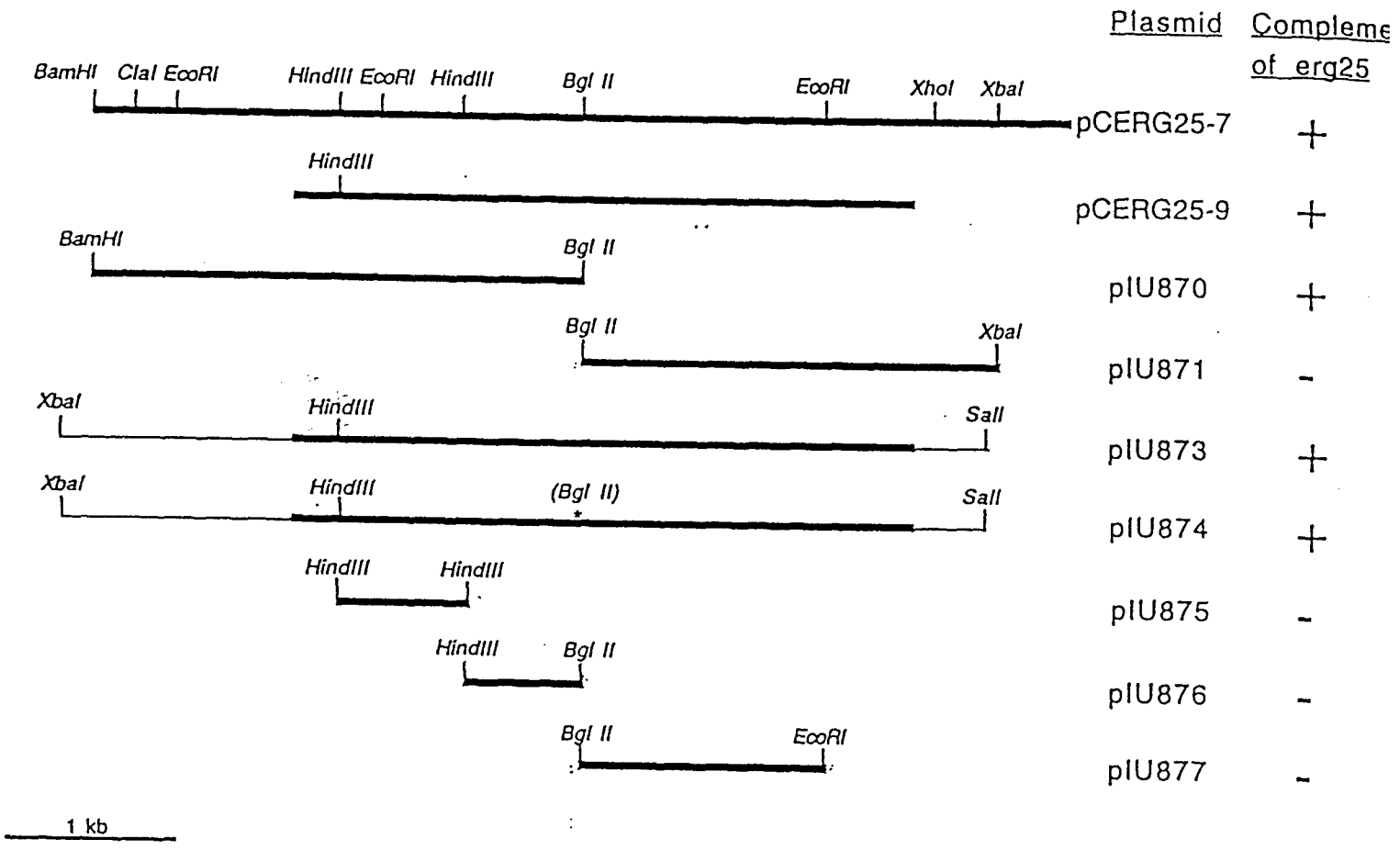

Figure 6. Restriction endonuclease sites on the *Candida albicans ERG25* genomic clones, pCERG25-7 and pCERG25-9. The ability of subclones pIU870 through pIU877 to complement an *erg25* recipient strain is indicated in the right column. Bold lines represent genomic DNA and thin lines represent plasmid pRS316 DNA.

```
   1  ttttgattcattaattgttatatttcaacatatacatattcctttattccttgatccttttttaaagtattcaatttat
  80  tatttatttgtttgtttgaagtttata ATG TCT TCC ATT AGT AAT GTT TAT CAT GAC TAT TCG AGT
                                  M   S   S   I   S   N   V   Y   H   D   Y   S   S     13

 147  TTT CTG AAT GCA ACT ACT TTT TCC CAA GTT TAT CAA AAT TTC AAT CAA TTA GAT AAT TTA
      F   S   N   A   T   T   F   S   Q   V   Y   Q   N   F   N   Q   L   D   N   L     33

 208  AAT GTT TTT GAA AAA TTA TGG GGG TCA TAT TAT TAT TAT ATG GCC AAT GAT TTA TTT GCT
      N   V   F   E   K   L   W   G   S   Y   Y   Y   Y   M   A   N   D   L   F   A     53

 269  ACT GGA TTA TTA TTT TTT TTA ACT CAT GAA ATT TTT TAT TTT GGT AGA TGT TTA CCA TGG
      T   G   L   L   F   F   L   T   H   E   I   F   Y   F   G   R   C   L   P   W     73

 330  GCT ATA ATT GAT AGA ATT CCT TAT TTT AGA AAA TGG AAA ATT CAA GAT GAA AAA ATC CCT
      A   I   I   D   R   I   P   Y   F   R   K   W   K   I   Q   D   E   K   I   P     93

 391  AGT GAT AAA GAA CAA TGG GAA TGT CTT AAA TCA GTT TTA ACA TCT CAT TTC TTA GTT GAA
      S   D   K   E   Q   W   E   C   L   K   S   V   L   T   S   H   F   L   V   E    113

 452  GCT TTC CCA ATT TGG TTT TTC CAT CCA TTA TGT CAA AAA ATT GGT ATT AGT TAT CAA GTA
      A   F   P   I   W   F   F   H   P   L   C   Q   K   I   G   I   S   Y   Q   V    133

 513  CCA TTC CCT AAA ATT ACT GAT ATG TTG ATT CAA TGG GCA GTA TTT TTT GTT TTG GAA GAT
      P   F   P   K   I   T   D   M   L   I   Q   W   A   V   F   F   V   L   E   D    153

 574  ACT TGG CAT TAT TGG TTT CAT AGA GGA TTA CAT TAT GGG GTT TTC TAT AAA TAT ATT CAT
      T   W   H   Y   W   F   H   R   G   L   H   Y   G   V   F   Y   K   Y   I   H    173

 635  AAA CAA CAT CAT AGA TAT GCT GCT CCA TTT GGA TTG GCA GCA GAA TAT GCT CAT CCA GTT
      K   Q   H   H   R   Y   A   A   P   F   G   L   A   A   E   Y   A   H   P   V    193

 696  GAA GTT GCC TTA TTA GGA TTG GGT ACG GTT GGT ATT CCG ATT GTT TGG TGT CTT ATC ACT
      E   V   A   L   L   G   L   G   T   V   G   I   P   I   V   W   C   L   I   T    213

 757  GGT AAC TTG CAT CTT TTC ACA GTT TCC ATT TGG ATC ATT TTA AGA TTA TTC CAA GCC GTT
      G   N   L   H   L   F   T   V   S   I   W   I   I   L   R   L   F   Q   A   V    233

 818  GAT GCT CAT TCC GGT TAT GAA TTC CCT TGG TCT TTA CAT AAT TTC TTG CCA TTT TGG GCT
      D   A   H   S   G   Y   E   F   P   W   S   L   H   N   F   L   P   F   W   A    253

 879  GGT GCT GAT CAT CAT GAT GAA CAT CAT CAT TAT TTC ATT GGT GGA TAC TCT TCA TCT TTT
      G   A   D   H   H   D   E   H   H   H   Y   F   I   G   G   Y   S   S   S   F    273

 940  AGA TGG TGG GAT TTC ATT TTG GAT ACC GAA GCT GGT CCA AAA GCT AAA AAG GGT AGA GAA
      R   W   W   D   F   I   L   D   T   E   A   G   P   K   A   K   K   G   R   E    293

1001  GAC AAA GTC AAA CAA AAT GTT GAA AAA TTA CAA AAG AAG AAC TTA TAG agagagaaagagtat
      D   K   V   K   Q   N   V   E   K   L   Q   K   K   N   L   *                    308

1065  atgtgtacaacttctcaatgtttgtaccactttcaatattaatactgtttatttttggttttatttaatatatatatc
1145  atatctattcatagtgctacat
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Figure 7. The DNA and amino acid sequences of the *Candida albicans ERG25* gene.

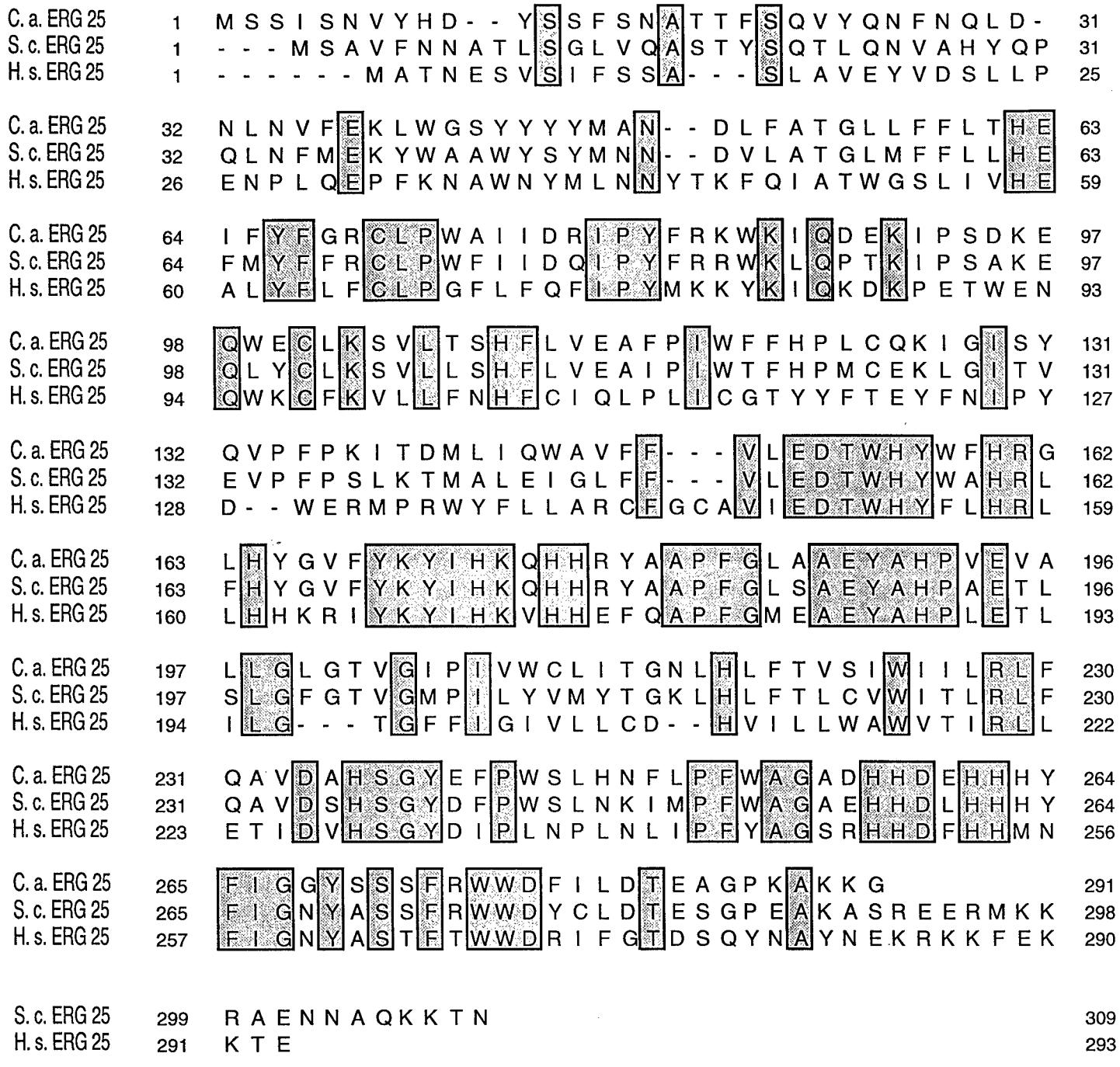

Figure 8. Alignment of the amino acid sequences of the C-4 sterol methyl oxidases from *Candida albicans* (c. a.), *Saccharomyces cerevisiae* (S. c.), and human (H. s.).

Appendix

STATEMENT OF WORK

Aim 2 Cloning and disruption of the C-24 Transmethylase gene (*ERG6*) of *Candida albicans*

-cloning by complementation of a *C. albicans* genomic library with a *Saccharomyces cerevisiae erg6* mutant
-confirmation of plasmid-borne phenotype (FOA,) GC/MS analysis
-characterization by restriction mapping and subcloning
-determination of essentiality by sequential disruption
-physiological characterization of *C. albicans ERG6* disruptions including susceptibility testing

Months 0-24

Aim 1 Isolation of C-4 demethylase mutants of *C. albicans*

Following isolation of the three genes for C-4 demethylation from *S. cerevisiae*:

Isolation of C-4 demethylase genes from *C. albicans*

-complementation of *S. cerevisiae* C-4 demethylase mutants with a genomic library from *C. albicans*
-confirmation of plasmid-borne phenotype (FOA), GC/MS analysis
-characterization by restriction mapping and subcloning
-gene disruption and allele replacement (sequential)
-analysis of essentiality
- sequencing of the *C. albicans* C-4 demethylase genes

Months 12-36

Aim 3 Suppressor analysis

isolation of suppressors of C4 demethylase mutants
characterization of suppressors
GC/MS analysis
sensitivity to inhibitors

Months 30-48

Recd 7/19/2000

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